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# BIOLOGICAL DEGRADATION OF CYANIDE BY NITROGEN-FIXING CYANOBACTERIA

by

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The study shows how nitrogen-fixing cyanobacteria (blue-green algae) can be used to treat small concentrations of free cyanides. The authors recommend application of a cyanobacteria-based process in the secondary treatment of cyanides that escape alkaline-chlorination. The document presents the step-by-step procedure they used to test their claims, as well as a results and discussion section, conclusion, and three-page bibliography. The authors note that while their process works, the kinetic data required to assess the feasibility of using cyanobacteria in the treatmentof cyanide wastes doesn't exist.


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## ABSTRACT

This study examined the ability of nitrogen-fixing Anabaena to biodegrade cyanide in batch reactors. Mixed second-order rate constants were obtained that described the biologically-mediated decrease in cyanide for reactors containing initial cyanide concentrations of 3 ppm. For Anabaena cultures not previously exposed to cyanide, the rate constants were a function of pH. Faster rates of cyanide biodegradation were observed at higher pH values. Anabaena cultures acclimated to the presence of cyanide had rate constants that were at least 10 times faster than rate constants for unacclimated cultures.

Mixed second-order rate constants were also obtained for the ability of nitrogenase, the enzyme normally responsible for nitrogen-fixation, to reduce hydrogen cyanide to methane and ammonia. In batch reactors with initial cyanide concentrations of 30 ppb, the rate constants for methane production were at least 10 times faster than expected based on literature values for nitrogen fixation, suggesting that nitrogenase will preferentially use hydrogen cyanide as a substrate as compared to molecular nitrogen. Also, the rate constants for methane production were of the same order of magnitude as the rate constants for total cyanide removal, indicating nitrogenase as an important mechanism for the biodegradation of trace concentrations of cyanide.

The magnitude of the cyanide biodegradation rate constants suggests that the utilization of nitrogen-fixing cyanobacteria in the treatment of cyanide wastes can be a feasible process in some applications, i.e., secondary or tertiary treatment at larger treatment facilities.




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# SECTION 1

# INTRODUCTION

The basic premise of this study was that the use of nitrogen-fixing cyanobacteria (blue-green algae) in the biological treatment of small concentrations of free cyanides (HCN and $CN^-$) can be a cost-effective alternative to existing treatment processes. A potential application of a cyanobacteria-based process would be in the secondary treatment of the free cyanides that escape alkaline-chlorination. Because the extent of cyanide oxidation in alkaline-chlorination is an equilibrium-driven phenomena, use of a microbial process to detoxify the last fraction of cyanide should result in lower alkaline-chlorination operating costs. Another application of a cyanobacteria-based process could be in the treatment of the cyanide associated with metal-cyanide complexes via a two-step process. The first-step would release cyanide from the metal-cyanide complexes by exposing the complexes to ultraviolet irradiation. In the second-step, cyanobacteria would detoxify the released cyanide.

The use of nitrogen-fixing cyanobacteria in the treatment of cyanide wastes is a new concept. There are several potential advantages associated with the use of nitrogen-fixing cyanobacteria in the treatment of small concentrations of cyanide. First, the biological treatment of cyanide with cyanobacteria should have much lower operating costs than alkaline-chlorination. The operating costs for the biological treatment of cyanide wastes with aerobic heterotrophs can be less than 10% the costs for alkaline-

chlorination (Green and Smith, 1972). The costs associated with providing aeration and with providing a supplemental energy source (organic substrate) for the maintenance of large amounts of aerobic heterotroph biomass make up a considerable portion of the total operating costs for the traditional biological treatment of hazardous wastes. Because cyanobacteria are photosynthetic, they do not require aeration for oxygen and do not require the presence of organic substrates to maintain biomass (Kobayashi and Rittmann, 1982). Thus, in terms of operating costs, the use of nitrogen-fixing cyanobacteria in the treatment of small amounts of cyanide should have an economic advantage over the use of heterotrophic bacteria, and, consequently, a significant economic advantage over alkaline-chlorination.

Second, nitrogen-fixing cyanobacteria have the ability to survive in low to moderate concentrations of hydrogen cyanide. Hydrogen cyanide is toxic because it inhibits the terminal cytochrome oxidase in respiration, which normally reduces oxygen to water. Cyanobacteria have several terminal oxidases--some of which are resistant to cyanide inhibition (Fogg, et al., 1973; Degn, et al., 1978; Peschek, 1980; Henry, 1981). Cyanobacteria also have several cyanide detoxification pathways, i.e., enzymatic pathways that transform free cyanides into a less toxic form (Castric, 1981; Higgins, et al., 1984). The most studied and perhaps the most important detoxification pathway is mediated by the enzyme rhodanese, which transfers a sulfur from a donating compound (e.g., thiosulfate) to cyanide to form thiocyanate (Westley, 1981). Other cyanide detoxification pathways result in the formation of amino acids (Solomonson, 1981). Thus, due to the presence of cyanide detoxification pathways and cyanide-resistant respiration, cyanobacteria can survive in solutions containing free cyanides. For example, Howe (1963, 1965) observed a

"luxuriant" growth of cyanobacteria on the filter stones of a biological reactor that was treating wastes containing 300 ppm cyanide.

Third, in addition to the above pathways, the nitrogen-fixing cyanobacteria can destroy hydrogen cyanide with the enzyme nitrogenase. While normally responsible for the reduction of molecular nitrogen (dinitrogen) to ammonia, nitrogenase can also reduce hydrogen cyanide to methane and ammonia (Hardy and Knight, 1967; Hardy and Burns, 1968; Biggins and Kelley, 1970; Haystead, et al., 1970; Hwang and Burris, 1972; Hwang, et al., 1973; Zumft and Mortenson, 1975; Stewart, 1980; Li, et al., 1982). In fact, nitrogenase will preferentially reduce hydrogen cyanide instead of its normal substrate, dinitrogen (Li, et al., 1982). Some researchers have proposed that the original role of the nitrogenase system was to detoxify the cyanide and cyanogen found in the primitive biosphere when the earth had a reducing atmosphere (Silver and Postgate, 1973; Postgate, 1982).

Despite the considerable amount of information indicating that cyanobacteria are able to survive in the presence of cyanide and are able to detoxify cyanide, the kinetic data required to assess the feasibility of utilizing cyanobacteria in the treatment of cyanide wastes does not exist. This study provides an initial assessment of the rate at which nitrogen-fixing cyanobacteria are able to degrade free cyanide. In particular, the mixed second-order rate constants for the biologically-mediated removal of cyanide by unacclimated cultures of Anabaena were determined in batch reactors. A second set of batch experiments determined the mixed second-order rate constants for the reduction of hydrogen cyanide by nitrogenase.

## SECTION 2

## CONCLUSIONS

In batch reactors with initial cyanide concentrations of 3 mg/L, the mixed second-order rate constants ($K_b$) for the removal of cyanide by nitrogen-fixing Anabaena cultures was a function of pH. The biodegradation rate constants increased with increasing pH for unacclimated Anabaena cultures, i.e., cultures not previously exposed to cyanide. At a pH of 8.4 and a temperature of 25°C, the observed rate constant in terms of chlorophyll (chl) concentration was $5.0 \cdot 10^{-6}$ L/(ug chl hr). When the pH was increased to 9.5, $K_b$ increased to $2.2 \cdot 10^{-4}$ L/(ug chl hr). The observed relationship between $K_b$ and pH suggested that the ratio of hydrogen cyanide to total free cyanide concentrations, $[HCN]/([HCN]+[CN^-])$, influenced biodegradation rates. Because HCN is much more toxic and inhibitory than $CN^-$, and because an increase in pH would reduce HCN concentrations, the faster biodegradation rates observed at higher pH values was probably due to a reduction in inhibition.

When previously exposed to cyanide, Anabaena biodegraded cyanide at a faster rate. The $K_b$ value for an acclimated Anabaena culture was 10 times greater than that for an unacclimated Anabaena culture. Thus, reactors operating under steady-state conditions should have faster cyanide removal rates than those observed in the batch experiments.

The mixed second-order rate constant for the reduction of cyanide to methane by nitrogenase ($K_n$) was determined in small gas-tight batch reactors with initial cyanide concentrations of 30 ug/L. A typical value of $K_n$ was

$2.6 \cdot 10^{-4}$ L/(ug chl hr), which was at least an order of magnitude greater than expected based on existing nitrogen-fixation kinetic data. The larger than expected $K_n$ supported the in vitro observation that nitrogenase will preferentially reduce hydrogen cyanide rather than its normal substrate of molecular nitrogen. Based on the amount of methane produced during the batch tests, nitrogenase activity reduced cyanide concentrations from 30 ug/L down to 20 ug/L. Because few cyanide destruction processes are able to attack cyanide at such low concentrations, the utilization of nitrogen-fixing cyanobacteria in the treatment of trace-levels of cyanide is worth further examination.

Based on the total cyanide biodegradation rate constants ($K_b$) obtained from batch experiments, use of nitrogen-fixing cyanobacteria in the secondary or tertiary treatment of cyanide wastes could be a feasible process, provided that the treatment process has adequate mean cell residence time. With a net specific growth rate of 0.8 $d^{-1}$, the unacclimated Anabaena culture can have faster growth kinetics and trace-contaminant removal rates than existing biological treatment processes. Because cyanobacteria are photosynthetic, the amount of biomass in a reactor is not a function of organic substrate concentration. Thus, the volumetric size of the reactors required to treat low concentrations of cyanide would be small compared to traditional aerobic processes for treating dilute wastes. For example, a chemostat model using conservative rate and biomass parameters predicted that a hydraulic retention time of only 5 days is required to reduce an influent cyanide concentration of 4 mg/L by 93 percent. In comparison, the 90 percent reduction of a 10 mg/L influent BOD concentration in an aerobic chemostat would require a hydraulic retention time of 21 days, based on typical BOD kinetic parameters (Metcalf

and Eddy, 1979). However, the required size of the BOD chemostat would be reduced if supplemental substrates were added to the reactor to increase biomass concentrations.

Because of the potential to attack trace-concentrations of cyanide and of the potential for low operating costs, the use of nitrogen-fixing cyanobacteria in the biological treatment of cyanide wastes may be an attractive alternative to exiting cyanide treatment technologies. However, the capital costs and the requirement for trained personnel associated with biological treatment processes, may make the cyanobacteria process unsuitable for small-volume cyanide-waste generators. The cyanobacteria process is probably better suited for larger cyanide treatment facilities.

## SECTION 3

## RECOMMENDATIONS

This study demonstrated the ability of nitrogen-fixing cyanobacteria to biodegrade low concentrations of free cyanides in batch reactors. Future studies need to examine the ability of the cyanobacteria to degrade cyanide under steady-state conditions and to determine the stability of the steady-state reactors to slight perturbations in cyanide concentrations. If such laboratory-scale experiments continue to demonstrate the attractiveness of utilizing nitrogen-fixing cyanobacteria in the treatment of low cyanide concentrations, then a pilot-scale study should be performed to determine the economic and technical feasibility of the process.

# SECTION 4

# MATERIALS AND METHODS

## EXPERIMENTAL OVERVIEW

The objectives of the study were 1) to determine the rates at which a cyanobacteria culture removed free cyanides ($HCN + CN^-$), and 2) to determine the rate at which HCN was reduced to methane by the activity of the enzyme nitrogenase. Rates of total cyanide removal were determined in a 1.3-liter reactor under batch conditions. Attempts were also made to determine total cyanide removal rates under continuous-feed conditions, i.e., in a chemostat. The methane production experiments were conducted in small 0.037-liter, gas-tight vials under batch conditions.

## CULTURE MEDIA

The cyanobacteria used in the cyanide degradation experiments were Anabaena sp. obtained from Carolina Biological Supply Co. (Burlington, NC, catalog number 151710). None of the cultures used in the study were axenic, i.e., none were free of bacteria.

The media used to maintain Anabaena cultures and used in the cyanide degradation experiments was the Hughes-Gorham-Zehnder media described in Allen (1973). The chemical composition of the media is shown on Table 1. To increase the buffering capacity of the media at pH values slightly greater than the $pK_a$ for hydrogen cyanide for media used in the batch experiments 0.186 grams of $HBO_3$ was added to each liter of media (3 mM) . Adjustments in pH were performed by the addition of either 1M NaOH or HCl. Prior to its use,

TABLE 1. CYANOBACTERIA CULTURE MEDIA

| Macronutrients | grams/liter |
|---|---|
| $K_2HPO_4$ | 0.369 |
| $MgSO_4 \cdot 7H_2O$ | 0.075 |
| $CaCl_2 \cdot 2H_2O$ | 0.036 |
| $Na_2CO_3$ | 0.020 |
| $NaSiO_3 \cdot H_2O$ | 0.058 |
| Citric Acid | 0.006 |
| EDTA | 0.001 |
| Ferric Citrate | 0.006 |
| Micronutrient Solution | 0.08 mL |

| Micronutrient Solution | grams/liter |
|---|---|
| $H_3BO_3$ | 3.10 |
| $MnSO_4 \cdot H_2O$ | 1.69 |
| $ZnSO_4 \cdot H_2O$ | 0.287 |
| $(NH_4)Mo_7O_{24} \cdot 4H_2O$ | 0.088 |
| $Co(NO_3)_2 \cdot 6H_2O$ | 0.0167 |
| $Na_2WO_4 \cdot 2H_2O$ | 0.33 |
| KBr | 0.119 |
| KI | 0.083 |
| $Cd(NO_3)_2 \cdot 4H_2O$ | 0.154 |
| $NiSO_4 \cdot 6H_2O$ | 0.138 |
| $V_2O_5$ | 0.018 |
| $Al_2(SO_4)_3 \cdot K_2SO_4 \cdot 24H_2O$ | 0.474 |

the media was autoclaved. Ferric citrate was autoclaved separately from the rest of the media to prevent the precipitation of ferric phosphates. Because no fixed-nitrogen was added to the media, the Anabaena had heterocysts and relied on the fixation of dinitrogen for their nitrogen needs.

Maintenance cultures were grown in autoclaved 100-mL Erlenmeyer flasks that were stoppered with cheese-cloth-wrapped cotton plugs. The maintenance cultures were illuminated by cool-white fluorescent light, and were not continuously agitated nor was air bubbled through the media. Probably due to $CO_2$ limitations, the Anabaena in the maintenance cultures had slow growth rates.

The Anabaena used in the total cyanide removal and the methane production batch tests were not directly transferred from maintenance culture to respective batch reactors. Instead, an inoculum from a maintenance culture flask was placed into a 500-mL gas wash bottle that contained fresh media. The media was agitated by the introduction of filtered air passing through a porous glass diffuser. In addition to providing agitation, the air bubbles released by the diffuser provided a continuous supply of $CO_2$ to the system. Anabaena in the gas wash bottle had rapid growth rates (doubling times on the order of 1 day). Upon reaching a desired biomass (chlorophyll) concentration, portions of the gas-wash-bottle cultures were transferred to the batch experiment reactors.

## EXPERIMENTAL PROCEDURES

### Growth Rate Experiments

Anabaena growth rates were determined in a New Brunswick Scientific (Edison, NJ) Bioflo Chemostat Model C32 with a Pyrex reaction vessel that held 1.3 liters of media. During the initial growth rate experiments, the reactor

was operated in batch mode (no continuous feed) and no cyanide was present. Temperatures were maintained at 25°C. Air was supplied at a rate of 15 L/hr. Increases in biomass were determined by monitoring the increase in chlorophyll-a (chl) concentrations with respect to time. The rate of increase in biomass was assumed to be first-order with respect to biomass, i.e,

$$\frac{dX}{dt} = u\,X \qquad (1)$$

in which X is the chlorophyll-a concentration (ug chl/L), t is time (hr), and u is the net specific growth rate ($hr^{-1}$). The value of u was obtained by determining the slope (least-squares fit) of the line produced when the natural logarithm of X (ln X) was plotted versus time. The amount of time required for the value of X to double was determined from

$$t_d = \frac{0.693}{u} \qquad (2)$$

in which $t_d$ is the doubling time (hr).

Subsequent growth rate experiments were attempted while operating the reactor as a chemostat (once-through reactor with continuous feed). Regardless of the feed flow rate, agitation speed, air flow rate, light intensity, and method of reactor start-up, steady-state chlorophyll concentrations were never obtained. As discussed in the following section, X values always decreased at a rate faster than would be predicted based on the dilution rate (feed flow rate divided by reactor volume).

Since the available flow-through reactor was unable to support steady-state populations of Anabaena in either the absence and presence of cyanide, the kinetic coefficients for the degradation of cyanide reported in this study were determined from short-term batch experiments.

## Total Cyanide Removal Experiments

### Objective and Apparatus--

The objective of the total cyanide removal experiments was to determine the mixed-second order rate constants for the biologically mediated removal of free cyanides from the 1.3-liter reactor operated in a batch mode. These batch experiments were of short duration (4 to 5 hours), so that during the course of the experiment any increases in Anabaena biomass due to growth would be negligible. Initial total cyanide concentrations were approximately 3000 ug CN/L. Water temperatures were maintained at 25°C. Filtered air was added to the reactor at a rate of 15 L/hr, and air exiting the reactor was drawn through a gas-wash bottle containing a 1 N solution of NaOH to capture any HCN that was volatilized from the reactor.

### Cyanide Mass Balances--

The two mechanisms responsible for the decrease in total free cyanide concentrations in the 1.3-L reactor were biodegradation of HCN and $CN^-$ and volatilization of HCN. Based on these two mechanisms, the mass balance equation for the batch reactor is

$$\frac{dS}{dt} = - K_b X_o S - K_L a \text{ ALPHA } S \qquad (3)$$

in which S is the concentration of free cyanide (HCN + $CN^-$) in the reactor (ug CN/L), $K_b$ is the mixed second-order rate constant for the biodegradation of free cyanides by the microorganisms (L/(ug chl hr)), $X_o$ is the concentration of Anabaena in the reactor at the start of the batch experiment (ug chl/L), $K_L a$ is the overall mass transfer coefficient for the movement of HCN from the liquid phase to the gas phase (1/hr), and ALPHA is the ratio of HCN to the total amount of free cyanide in solution,

$$\text{ALPHA} = \frac{[\text{HCN}]}{[\text{HCN}] + [\text{CN}^-]} . \qquad (4)$$

The ALPHA parameter must be included in the volatilization term, because HCN is the only form of free cyanide that is subject to volatilization. ALPHA is a function of the $pK_a$ of HCN and of the pH of the solution. Because the $pK_a$ of HCN at 25°C is 9.3, values of ALPHA can be obtained from (Snoeyink and Jenkins, 1980)

$$\text{ALPHA} = \frac{10^{-pH}}{10^{-pH} + 10^{-9.3}} \qquad (5)$$

in which pH is $-\log[H^+]$ and log is the base 10 logarithm.

The rate of increase in the mass of HCN collected in the gas-wash bottle as $CN^-$ can be described by

$$\frac{dM}{dt} = V\ K_L a\ \text{ALPHA}\ S \qquad (6)$$

in which M is the total mass of free cyanide in the gas-wash bottle (ug CN) and V is the volume of the biological batch reactor (1.3 L).

Kinetic Parameter Determination--

The data obtained from each batch experiment included 1) the initial free cyanide concentration ($S_o$), 2) the initial Anabaena biomass in terms of chlorophyll-a concentration ($X_o$), 3) the total cyanide concentration (S) and pH of each sample collected from the reactor, and 4) the mass of cyanide found in the gas-wash bottle at the end of the batch experiment ($M_f$). The value of the overall mass transfer coefficient ($K_L a$) for stripping HCN out of the batch reactor was not known. Batch volatilization experiments conducted with sterile media indicated that $K_L a$ values changed significantly between replicates, despite the controls for the air flow rate and the agitation rate

being set at the same values. Thus, both $K_b$ and $K_La$ had to be determined from the experimental data for each batch test--$S_o$, $X_o$, S(t), pH(t), and $M_f$.

The values of $K_b$ and $K_La$ for each batch test were obtained via a trial and error algorithm utilizing the Runge-Kutta method to solve equation (3). First, an approximate value of $K_La$ was determined by setting $K_b$ to zero and finding the $K_La$ value that predicted the amount of cyanide collected in the gas-wash bottle at the end of the batch experiment ($M_f$). This approximate value of $K_La$ accounted for changes in pH. Second, this approximate value of $K_La$ was increased and $K_b$ values were increased until the Runge-Kutta model approximated the observed decrease in total cyanide concentration (S) with time. Third, values of $K_La$ and $K_b$ were then adjusted to yield the best description of observed $M_f$ and S(t) values.

Methane Formation Experiments

Objective and Apparatus--

The objective of the methane experiments was to determine the mixed-second order rate constant for the conversion of HCN by nitrogenase to methane and ammonia by monitoring the increase in headspace methane concentrations in gas-tight reactors that were operated in a batch mode. The batch tests were of short duration (1 to 2 hours), so that increases in Anabaena biomass or changes in environmental conditions during the course of the experiment would be insignificant. The Anabaena used in the methane production experiments had not previously been exposed to cyanide. Initial total cyanide concentrations were approximately 30 ug CN/L. To minimize the volatilization of HCN, initial pH values were adjusted to 10. Water temperatures during the experiments were 25°C. Methane concentrations were determined by injecting 100 uL samples of headspace gas into a Hewlett-Packard 5840A gas chromatograph with a flame-

ionization detector (FID).

The reactors used in methane experiments consisted of a 40-mL Wheaton serum bottles fitted with a Supelco Mini-Valve stopper. The stopper was a combination value and septum that provided a means of sampling the headspace of the bottle, while being gas-tight when the headspace was not being sampled. To maintain a uniform distribution of Anabaena in the media, a small Teflon-coated magnetic stir-bar was placed inside the bottle and the bottle was placed on top of a stirring plate. During the batch experiments, the bottle contained 25 mL of media and had a headspace volume of 12 mL. The stem of the stopper occupied the remaining 3 mL.

Kinetic Parameter Determination--

Each mole of methane appearing in the headspace of the serum bottle was assumed to be produced from the reduction of one mole of hydrogen cyanide to methane and ammonia by nitrogenase. Thus, the molar concentration of methane in the headspace corresponds to a mass concentration of cyanide that was reduced by nitrogenase,

$$P = [CH_4] \frac{MW_{CH_4}}{MW_{CN}} \frac{V_{gas}}{V_{water}} \qquad (7)$$

in which P is the cyanide concentration in the water phase corresponding to the mass of methane produced (ug CN/L), $[CH_4]$ is the molar concentration of methane in the headspace gas (umoles $CH_4$/L of gas), $MW_{CH_4}$ is the molecular weight of methane, $MW_{CN}$ is the molecular weight of the cyanide function group, $V_{gas}$ is the volume of the headspace (0.012 L), and $V_{water}$ is the volume of the water phase in the serum bottle (0.025 L). Because P represented another way of describing methane concentration (the product of nitrogenase activity), the value of P increased with time during the methane batch tests.

The rate of methane production by nitrogenase was assumed to follow mixed second-order kinetics,

$$\frac{dP}{dt} = K_n X_o S \qquad (8)$$

in which P is the water-phase concentration of cyanide corresponding to the mass of methane produced (ug CN/L), t is time (hr), $K_n$ is the mixed second-order rate constant for the production of methane by nitrogenase expressed in terms of chlorophyll-a concentration (L/(ug chl hr)), $X_o$ is the concentration of Anabaena at the start of the batch test (ug chl/L), and S is the total cyanide concentration at time t (ug CN/L).

Because nitrogenase was not the only biological mechanism for reducing cyanide concentrations (S) in the vials, the determination of $K_n$ required that the decrease in S in equation (8) reflect these additional mechanisms. The total rate at which S decreases with time due to biological activity can be described by

$$\frac{dS}{dt} = - K_b X_o S \qquad (9)$$

in which $K_b$ is the mixed second-order rate constant for the biodegradation of free cyanides by Anabaena cultures (L/(ug chl hr)). Integration of equation (9) yields

$$S = S_o \exp[-K_b X_o t] \qquad (10)$$

in which $S_o$ is the initial cyanide concentration (ug CN/L). Substitution of equation (10) for S in equation (8) yields the following equation:

$$\frac{dP}{dt} = K_n X_o S_o \exp[-K_b X_o t] \; . \qquad (11)$$

Integration of equation (11) from t=0 and $P=P_o=0$ to t=t and P=P produces

$$P = \frac{K_n S_o}{K_b} \{1 - \exp[-K_b X_o t]\} \quad (12)$$

which describes methane concentration as a function of time.

The goal of the methane batch experiments was to determine the value of $K_n$ for the nitrogen-fixing Anabaena culture. For each batch test, all of the parameters in equation (12) were known, except for $K_n$. The value of the overall biodegradation rate constant ($K_b$) was determined from the batch experiments examining total cyanide removal. When an independent variable (RHS) is defined for each sampling time by

$$RHS = \frac{S_o}{K_b} \{1 - \exp[-K_b X_o t]\} , \quad (13)$$

equation (12) is reduced to a linear relationship,

$$P = K_n RHS . \quad (14)$$

Thus, the value of $K_n$ for each batch test was calculated from the slope of a linear least-squares fit of the P(t) and RHS(t) data points collected during the batch test. The y-intercept for equation (14) should equal zero, because $P_o=0$.

ANALYTICAL PROCEDURES

Cyanide and chlorophyll measurements followed the procedures presented in Standard Methods (APHS, 1980).

Prior to analysis, cyanide samples were distilled in a commercially-available cyanide distillation apparatus. In the boiling flask, the cyanide sample was subjected to acidic conditions and to a magnesium chloride reagent. This solution was refluxed for 1 hour. The volatilized hydrogen cyanide was collected in a gas-wash bottle containing 1.25 N NaOH solution.

For samples with cyanide concentrations greater than 1 mg CN/L, the

cyanide concentrations in the alkaline distillate were determined by the titrimetric method. The distillate was titrated with a standard silver nitrate ($AgNO_3$) solution to form a soluble cyanide complex, $Ag(CN^-)_2$. As soon as all the CN had been complexed and a small excess concentration of $Ag^+$ had been added, the excess $Ag^+$ was detected by the silver-sensitive indicator, paradimethylaminobenzalrhodanine, which turned from a yellow to salmon color (APHS, 1980).

For samples with concentrations less than 1 mg CN/L, the cyanide content of the alkaline distillate was determined by the colorimetric method. First, the pH of a distillate sample was adjusted to approximately 8 with the addition of a phosphate buffer. Second, chloramine-T was added, which converted the $CN^-$ in the distillate to CNCl. Third, with the addition of a pyridine-barbituric reagent, the CNCl formed a red-blue dye. The absorbance of the aqueous dye at 578 nm was linearly proportional to CN concentration in the distillate (APHS, 1980).

The concentration of chlorophyll-a in a sample was used as an indicator of Anabaena biomass concentration. Chlorophyll-a concentrations were determined by the cold acetone extraction method. A known volume of the cyanobacteria suspension with a small amount of $MgCO_3$ was drawn through a 0.45-um membrane filter. The filter was placed was placed into a centrifuge tube, and a small known volume of a 90% acetone ($v/v_{water}$) solution was added to the centrifuge tube. The acetone solution was allowed to extract the chlorophyll from the Anabaena for 24 hr at a temperature of 4°C. After centrifugation, the chlorophyll-a concentration in the supernatant was calculated from the supernatant's absorbances at 663, 645, and 630 nm (APHS, 1980). Absorbances were measured with a Beckmann Spectrophotometer.

## SECTION 5

## RESULTS AND DISCUSSION

### GROWTH RATE DETERMINATION

For batch growth experiments conducted at pH values ranging from 8.0 to 9.5 in the absence of cyanide, the average net specific growth rate (u) of the Anabaena was 0.832 $d^{-1}$, which corresponded to a doubling time ($t_d$) of 20 hr. The fastest observed net growth rate was 1.07 $d^{-1}$ ($t_d$=15.6 hr); the growth curve for this batch experiment is shown on Figure 1. These growth rate values were comparable to growth rates described by others (Stewart, 1977; Fay, 1983; Bothe, et. al., 1984).

The Anabaena did not survive in maintenance cultures buffered at pH values of 10 or greater.

While the batch growth experiments yielded actively growing Anabaena populations, attempts to develop steady-state populations of Anabaena in a chemostat failed. The chemostat studies were performed in the same reactor and under the same environmental conditions as the batch growth experiments, except that a continuous feed was added to the reactor. When the reactor was subjected to a continuous feed, the concentration of chlorophyll in the reactor decreased at a rate faster than would be predicted based on the hydraulic dilution rate (D) for the reactor.

A typical response between chlorophyll concentration and time after the start of feed flow is shown on Figure 2. After 22 hr of flow, the chlorophyll concentration in the once-through reactor increased from 220 to 300 ug chl/L.

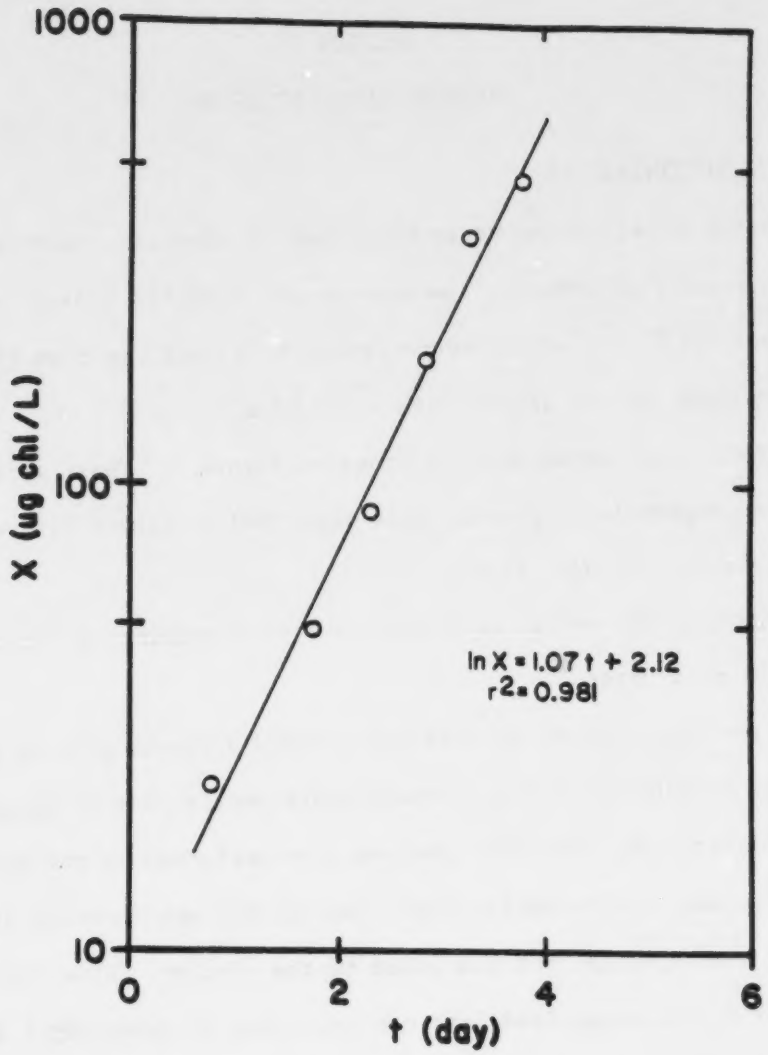


Figure 1. Logarithmic increase in Anabaena biomass (X) as function of time, during a batch experiment.

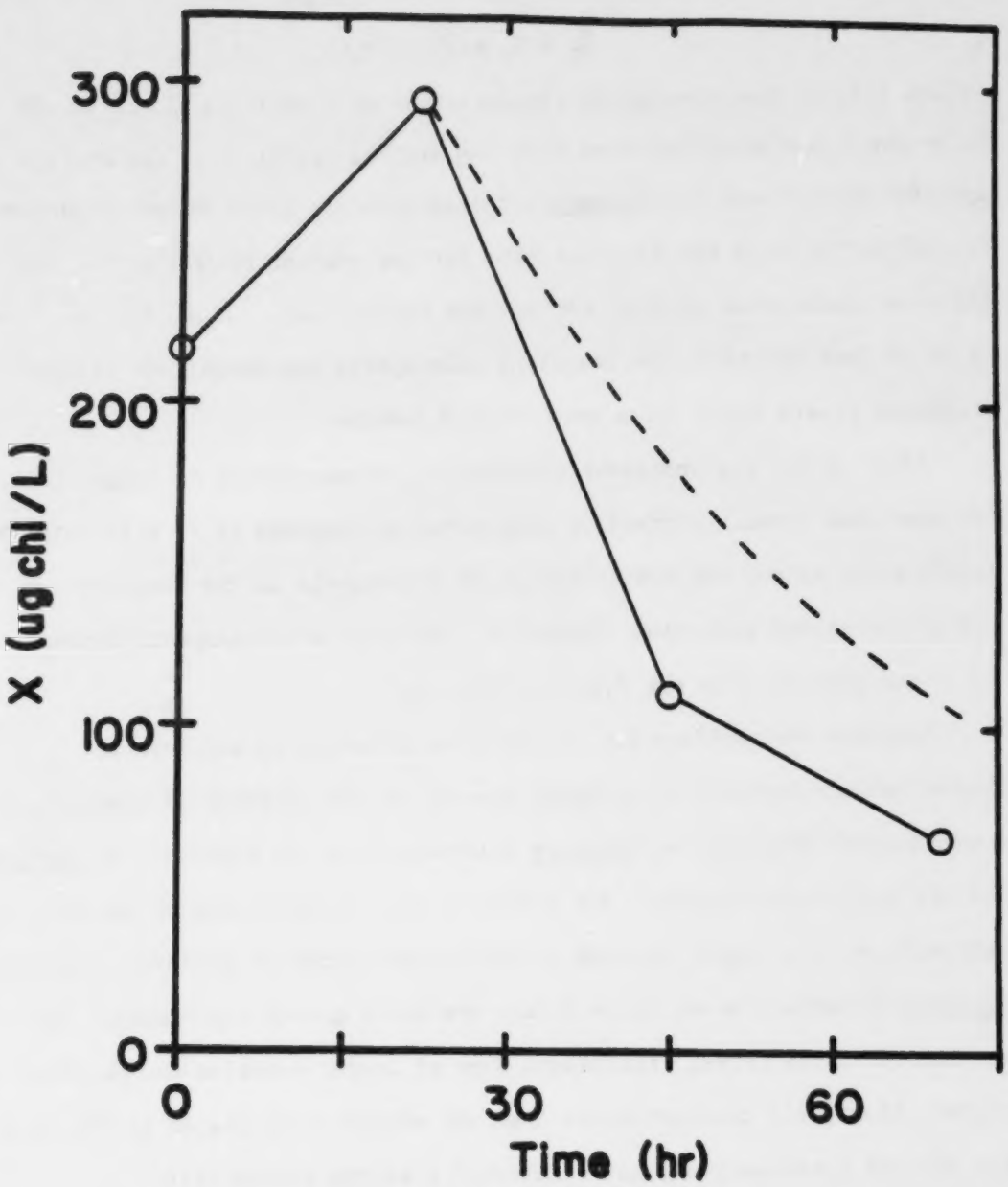


Figure 2. Changes in Anabaena biomass (X) versus time after the start of feed flow through the reactor. The dotted line represents the expected decrease in X if dilution was the only removal mechanism.

This biomass increase was equal to the increase predicted by the average net specific growth rate (u) minus the dilution rate (D), i.e.,

$$X_t = X_o \exp[(u-D)\ t] \qquad (15)$$

in which $X_t$ is the chlorophyll concentration at time t (ug/L), $X_o$ is the chlorophyll concentration when flow was started (ug/L), u is the average net specific growth rate for Anabaena obtained from the batch growth experiments (0.0347 $hr^{-1}$), D is the dilution rate for the reactor (0.0211 $hr^{-1}$), and t is the time since start of flow through the reactor (hr). Thus, for the first 22 hr of feed addition, the transient chlorophyll concentrations followed the predicted growth curve for a once-through reactor.

After 22 hr, the transient chlorophyll concentrations no longer followed the predicted trend. Instead of continuing to increase or of plateauing at a steady-state value, the concentration of chlorophyll in the once-through reactor decreased with time (Figure 2). The rate of chlorophyll decrease was 1.5 times greater than the dilution rate (D).

Possible explanations for the observed decrease in chlorophyll concentrations included an Anabaena die-off or the presence of a selective mechanism for removing the Anabaena trichomes from the reactor. An Anabaena die-off could have occurred, due either to cell lysis caused by the lack of nutrients or to a rapid increase in the concentration of grazers. Because Anabaena die-offs did not occur during the batch growth experiments, and because the batch growth experiments were of longer duration and obtained higher chlorophyll concentrations than the situation displayed in Figure 2, the die-off hypothesis was not considered a strong possibility.

The selective removal mechanism explanation was based on the fact that the flow exited from the reactor through an overflow weir. Any mechanism that

accumulated Anabaena in the neustron or in the upper regions of the reactor would have allowed the chlorophyll concentrations to decrease at a rate faster than predicted by the dilution rate. Possible mechanisms included the entrainment of the filamentous Anabaena on rising air bubbles and the buoyancy conferred on Anabaena due to the presence of gas vesicles. An interesting observation was that significant portions of the Anabaena collected in the overflow-collection vessel were buoyant. However, the observed buoyancy could have arisen from the different environmental conditions provided in the overflow-collection vessel.

Regardless of the mechanism, it was not possible to establish steady-state conditions in the once-through reactor either in the presence of or in the absence of cyanide. Chlorophyll concentrations always decreased at a rate faster than predicted by the dilution rate, despite variations in feed flow rate, agitation speed, air flow rate, light intensity, and method of reactor start-up. Because the available reactor could not function as a chemostat for the Anabaena cultures, the kinetic coefficients for the degradation of cyanide were determined from short-term batch experiments.

## TOTAL CYANIDE REMOVAL RATES

### Limitations of Batch Experiments

The mixed second-order rate constants ($K_b$) describing the biologically-mediated decrease in total cyanide concentrations by the Anabaena cultures were determined via short-term batch experiments. The initial objective of the batch experiments was to provide kinetic information for the setup of the chemostat experiments. With inability of the available once-through reactor to establish steady-state conditions, the results of the batch experiments, as the only source of kinetic information, became more important than originally

intended.

The short-term batch experiments were performed with Anabaena cultures that had not previously been exposed to cyanide. Because the cultures were not acclimated to the presence of cyanide (i.e., the optimal concentrations of cyanide degrading enzymes were not developed), the reported rate constants for total cyanide removal are probably slower than would be observed for acclimated Anabaena cultures. Another reason why the reported rate constants underestimate cyanide removal potential is that the maximum inhibitory effects of cyanide on respiration and enzyme activity were expressed during a batch test (i.e., protection mechanisms and pathways were not allowed to become fully operational). Thus, a steady-state tertiary cyanide treatment process utilizing Anabaena should have faster rate constants for the degradation of cyanide than the reported values.

Rate Constants for Total Cyanide Removal

The two mechanisms responsible for reducing total cyanide concentrations during a batch test were biodegradation and volatilization. The length of the batch tests listed on Table 2 was 4 hours, with the exception of batch test 1 which was 5 hours long. The extent of biodegradation and volatilization varied considerably between the batch tests. With the exception of batch test 1, the extent of cyanide loss due to volatilization increased as the time averaged pH value decreased. For example, for batch test 2 with a time-averaged pH value of 8.44, 1112 ug CN was volatilized, while the amount of cyanide volatilized during batch test 3 with a time-averaged pH of 9.5 was 138 ug CN. This was the expected result, because as pH dropped the fraction of cyanide existing as volatile hydrogen cyanide increased. In contrast, the extent of biodegradation increased as the time averaged pH value increased

TABLE 2. EXTENT OF BIODEGRADATION AND VOLATILIZATION IN SELECTED BATCH EXPERIMENTS

| Batch Number | Time Ave. pH | Initial Biomass $X_o$ (ug chl/L) | Total Mass CN Removed (ug CN) | Volatilized Mass of Cyanide (ug CN) | Biodegraded Mass of Cyanide (ug CN) |
|---|---|---|---|---|---|
| 1 | 9.59 | 412 | 752 | 534 | 218 |
| 2 | 8.44 | 4926 | 1188 | 1112 | 76 |
| 3 | 9.50 | 203 | 443 | 138 | 305 |
| 4* | 9.00 | 560 | 360 | 324 | 36 |
| 5** | 9.00 | 367 | 583 | 410 | 173 |

* media for this batch test included 0.5 ppm thiosulfate

** re-exposure of biomass in batch test #4 to cyanide and thiosulfate

(for this observation ignore batch test 5, which is a batch test examining the potential for acclimation). For example, at a time-averaged pH of 8.44 (batch test 2), biodegradation accounted for the removal of 76 ug CN. At a time-averaged pH of 9.5 (batch test 3), biodegradation accounted for the removal of 305 ug CN. Batch test 3 had a greater extent of biodegradation than batch test 2, despite it having only 4% of the biomass. These observations suggest that pH played an important role in determining rate of total cyanide removal, whether the removal mechanisms was predominantly by volatilization or by biodegradation.

The biodegradation and volatilization rate constants in equation (3) were determined from a computer algorithm. The resulting rate constants provided

the simultaneous best fit of two sets of data: the decrease in cyanide concentration with time in the reactor and the mass of cyanide collected in the gas-wash bottle. The calculated $K_b$ and $K_La$ values for batch tests 1 through 4 are listed on Table 3. As shown on Figure 3 for batch test 3, the calculated values of $K_b$ and $K_La$ described the decrease in total cyanide concentration. Similar fits of the data were obtained for the other batch tests.

TABLE 3. OVERALL VOLATILIZATION RATE CONSTANTS ($K_La$) AND MIXED SECOND-ORDER RATE CONSTANTS ($K_b$) FOR TOTAL CYANIDE REMOVAL BY UNACCLIMATED ANABAENA CULTURES IN SELECTED BATCH EXPERIMENTS

| Batch Number | Time Averaged pH | Time Averaged ALPHA | $K_La$ ($hr^{-1}$) | $K_b$ [L/(ug chl hr)] |
|---|---|---|---|---|
| 1 | 9.59 | 0.339 | 0.17 | $8.5 \cdot 10^{-5}$ |
| 2 | 8.44 | 0.879 | 0.22 | $5.0 \cdot 10^{-6}$ |
| 3 | 9.50 | 0.387 | 0.052 | $2.2 \cdot 10^{-4}$ |
| 4* | 9.00 | 0.666 | 0.075 | $8.0 \cdot 10^{-6}$ |

* media for this batch test included 0.5 ppm thiosulfate

Examination of Table 3 indicates that the fitted values of $K_b$ and $K_La$ varied considerably between the 4 batch tests. Because the effect of temporal changes in pH on volatilization rates were accounted for by the ALPHA term in equation (3), the 4-fold variation in the calculated $K_La$ values was due to differences in aeration rates and agitation intensities. These differences existed, despite the mechanical settings on the experimental reactor being the same for each batch test.

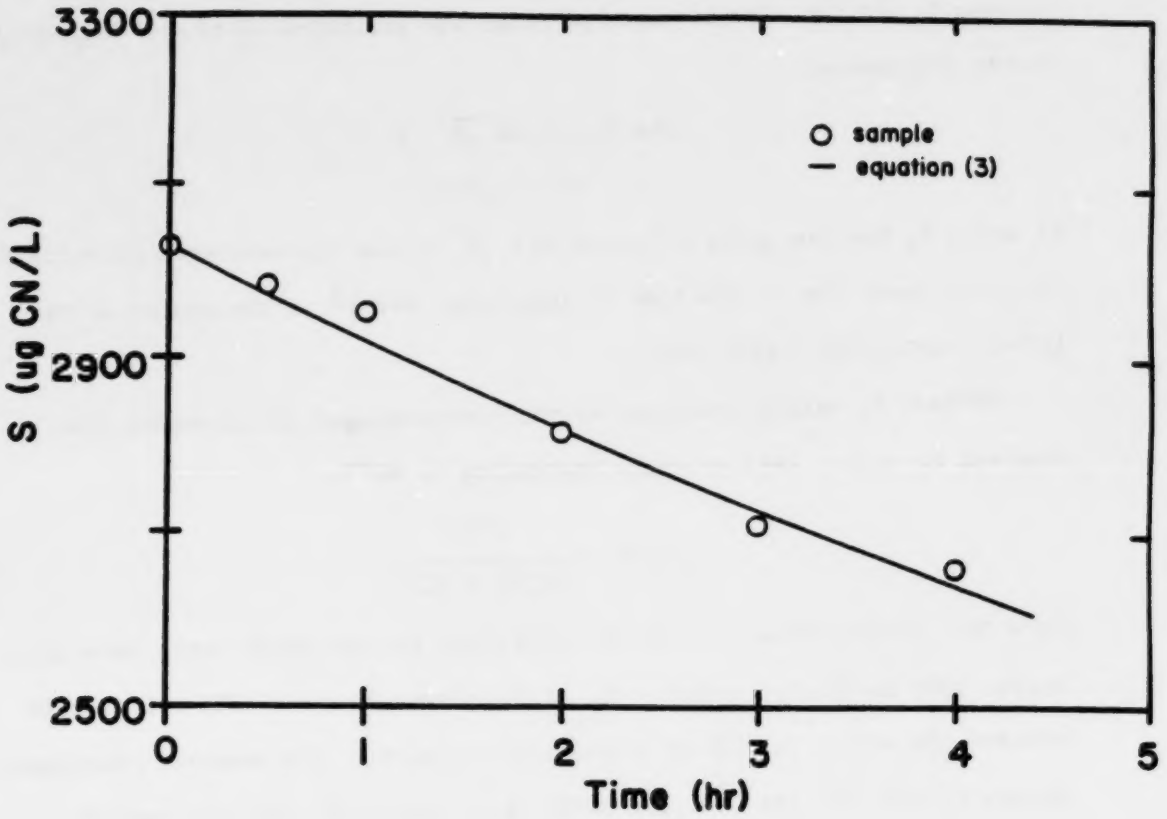


Figure 3. Decrease in total cyanide concentration (S) during batch test number 3. The line represents the numerical solution of equation (3) using the $K_b$ and $K_L a$ values on Table 3.

The mixed second-order biodegradation rate constants ($K_b$) for the unacclimated Anabaena cultures ranged from $5.0 \cdot 10^{-6}$ to $2.2 \cdot 10^{-4}$ L/(ug chl hr). This observed 44-fold variation in the $K_b$ values appeared to be a function of pH. As shown on Figure 4, the biodegradation rate constant increased as the time-averaged pH for each batch test increased. The observed relationship between $K_b$ and the time-averaged pH value was approximated by the following linear regression:

$$\log K_b = 1.44 \, \overline{pH} - 17.7 \qquad (16)$$

$$r^2 = 0.836$$

in which $K_b$ has the units L/(ug chl hr), $\overline{pH}$ is the time-averaged pH value for the batch test, log is the base 10 logarithm, and $r^2$ is the square of the linear regression coefficient.

Because $K_b$ values increased as the time-averaged pH increased, the observed $K_b$ values were probably responding to ALPHA,

$$\text{ALPHA} = \frac{[HCN]}{[HCN] + [CN^-]} . \qquad (4)$$

Since the initial total cyanide concentration for the batch tests were similar (aprox. 3000 ug CN/L), comparing $K_b$ to the time-averaged ALPHA value would indicate the effect of HCN on biodegradation rates. The observed relationship between $K_b$ and the time-averaged ALPHA value suggested that for smaller concentrations of HCN the rate of total cyanide biodegradation by the Anabaena cultures increased (Figure 5). The relationship between $K_b$ and ALPHA was approximated by the following linear regression:

$$\log K_b = -2.91 \, \text{ALPHA} - 2.88 \qquad (17)$$

$$r^2 = 0.862$$

in which ALPHA was calculated from the time-averaged pH for each batch test.

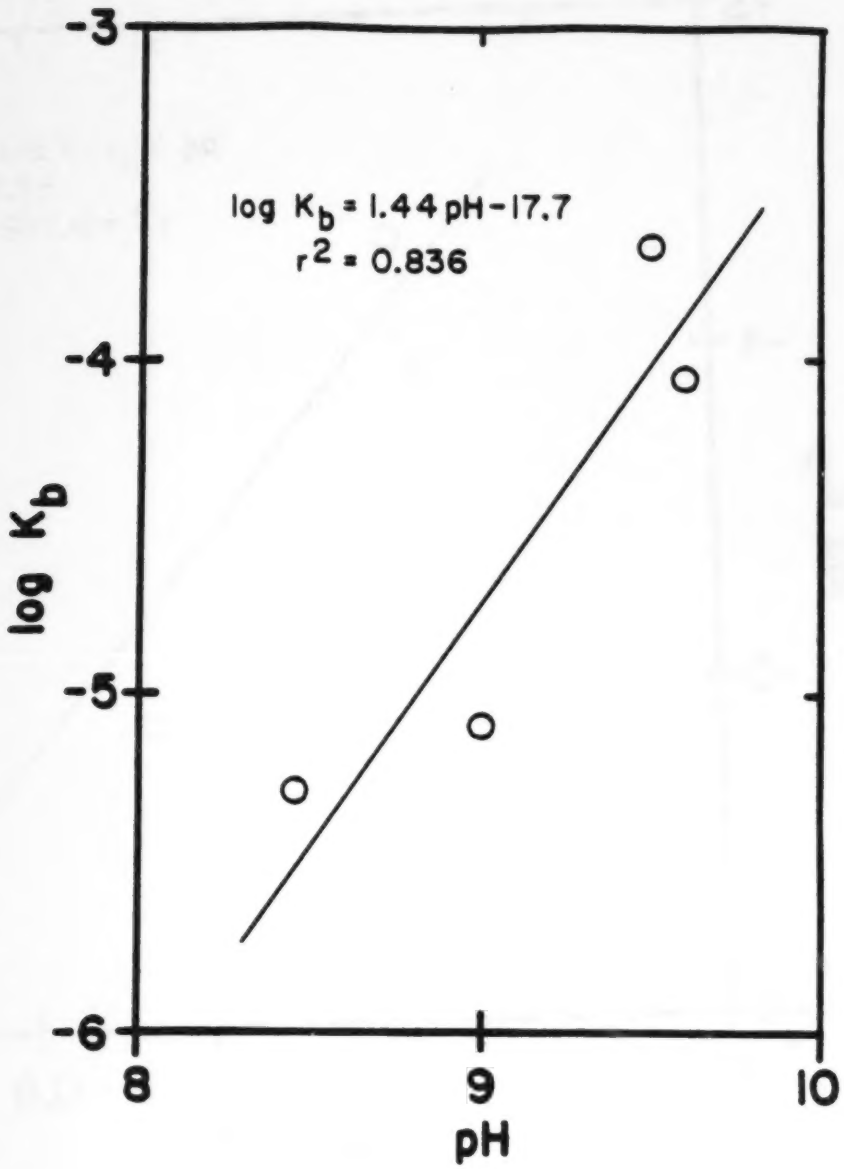


Figure 4. Relationship between log $K_b$ [L/(ug chl hr)] versus time-averaged pH.

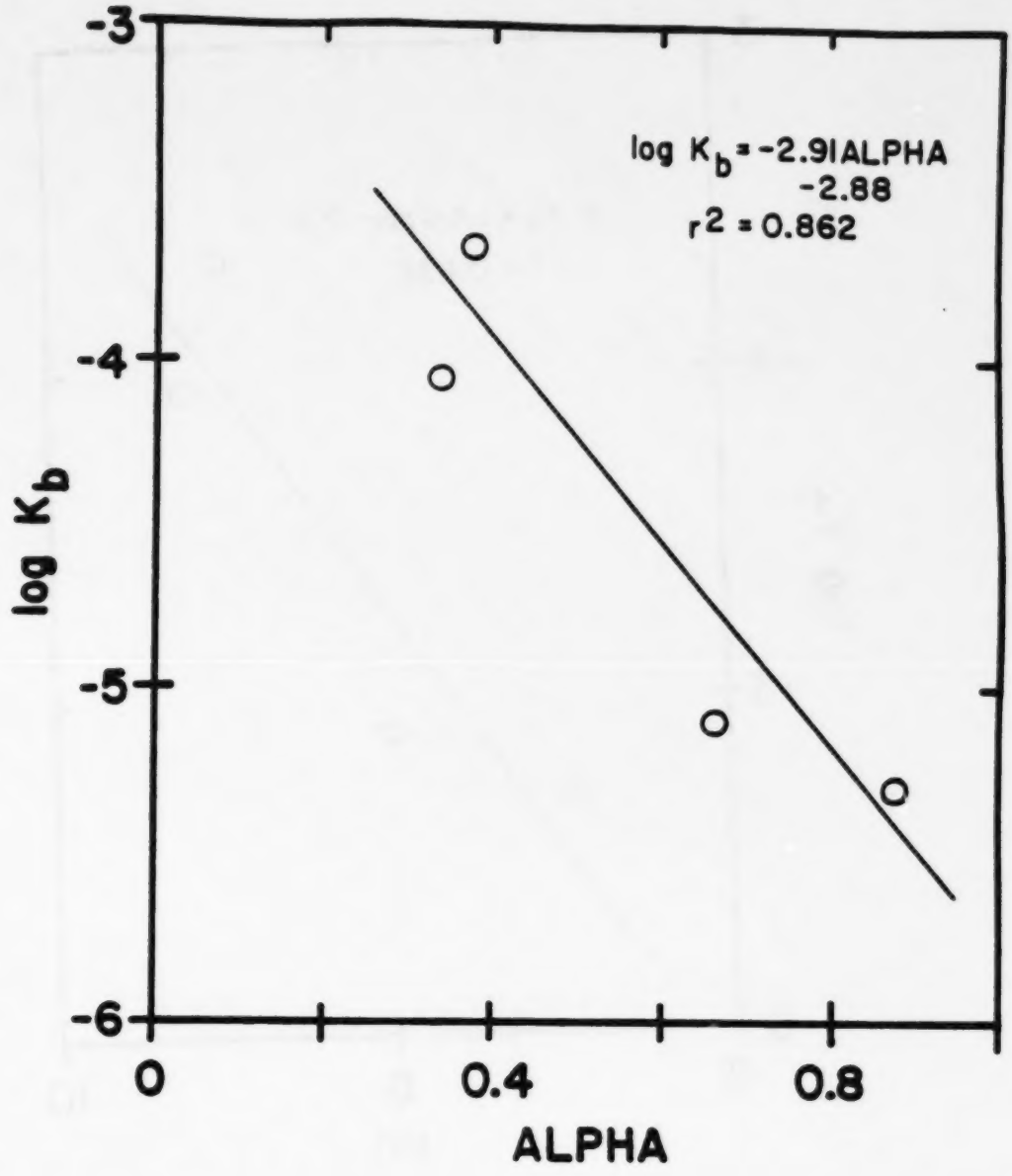


Figure 5. Relationship between log $K_b$ [L/(ug chl hr)] versus time-averaged ALPHA.

The rate at which unacclimated Anabaena cultures reduced total cyanide concentrations was optimized by reducing the fraction of the cyanide that existed as HCN (reduced ALPHA value). This observation is consistent with the concept that HCN is more toxic and inhibitory than $CN^-$ (Doudoroff, et al., 1966; Doudoroff, 1976). The ALPHA value is smallest at pH values greater than the $pK_a$ for HCN. Operating a reactor at pH values above the $pK_a$ for HCN also reduces the rate of volatilization. These factors suggest that the biodegradation efficiency of a cyanide-treatment process based on nitrogen-fixing cyanobacteria would increase as the pH of the system is increased. This improvement in reactor efficiency with pH would occur only up to pH values of 10, because Anabaena culture did not survive at pH values greater than 10.

The maximum observed $K_b$ for the degradation of cyanide by unacclimated Anabaena cultures is larger than the mixed second-order rate constants for existing biological treatment processes. Converting the observed $K_b$ for batch test 3 into units of L/(mg VSS d) where VSS is volatile suspended solids, the maximum observed rate constant was 0.0195 L/(mg VSS d). In comparison, the biodegradation of acetate by methanogens (i.e., anaerobic heterotrophs that produce methane) can have a mixed second-order rate constant of 0.0054 L/(mg VSS d) (Rittmann and McCarty, 1980). Thus, the biodegradation of cyanide by unacclimated Anabaena cultures can have faster rate constants than the biodegradation of acetate by methanogens. The observed net specific growth rate for Anabaena under optimum conditions was 0.832 $d^{-1}$, while the maximum specific growth rate for the acetate-utilizing methanogens was reported as 0.25 $d^{-1}$ (Rittmann and McCarty, 1980). Because Anabaena can have faster growth rates and contaminant removal rates than methanogens, and

because successful wastewater treatment facilities have been developed using methanogens, there appears to be no kinetic limitation for the development of biological treatment processes utilizing Anabaena. However, other factors may limit process development, such as construction costs. As with the anaerobic treatment of wastes with suspended microorganisms, large reactors may be required to provide adequate cyanide removal and to ensure process stability.

Effect of Acclimation on Cyanide Removal Rates

By re-exposing an Anabaena culture to cyanide, the impact of acclimation on biodegradation rates was assessed. Batch test 5 was a repeat of batch test 4 (Table 2). Thiosulfate (0.5 ppm) was added to the culture media used in batch test 4 to induce the production of rhodanese, the enzyme that catalyzes the formation of thiocyanate from free cyanide and thiosulfate. The addition of thiosulfate was assumed to have no effect on the $K_b$ value for batch test 4. However, the induction of rhodanese and other detoxification pathways during batch test 4, were expected to increase the rate of total cyanide removal during batch test 5. Comparison of the $K_b$ values for batch tests 4 and 5 would indicate the difference in cyanide biodegradation kinetics between unacclimated and acclimated Anabaena cultures.

Several hours after completion of batch test 4, aeration and agitation were shut off in the experimental reactor. The Anabaena was allowed to settle overnight. The next morning the supernatant was siphoned off and replaced with new culture media containing thiosulfate. Aeration and agitation were restarted. Two hours later, the acclimated Anabaena culture was subjected to a second cyanide batch experiment.

The acclimated Anabaena culture reduced total cyanide concentrations 12 times faster than the unacclimated Anabaena culture (Table 4). The acclimated

TABLE 4. COMPARISON OF MIXED SECOND-ORDER RATE CONSTANTS ($K_b$) FOR TOTAL CYANIDE REMOVAL BY UNACCLIMATED (BATCH NUMBER 4) AND ACCLIMATED (BATCH NUMBER 5) ANABAENA CULTURES IN PRESENCE OF THIOSULFATE

| Batch Number | Time Averaged pH | Time Averaged ALPHA | $K_La$ ($hr^{-1}$) | $K_b$ [L/(ug chl hr)] |
|---|---|---|---|---|
| 4 | 9.00 | 0.666 | 0.075 | $8.0 \cdot 10^{-6}$ |
| 5 | 9.00 | 0.666 | 0.085 | $1.0 \cdot 10^{-4}$ |

Anabaena culture (batch test 5) had a mixed second-order rate constant ($K_b$) of $1.0 \cdot 10^{-4}$ L/(ug chl hr), while $K_b$ for the unacclimated Anabaena culture (batch test 4) was $8.0 \cdot 10^{-6}$ L/(ug chl hr).

Thus, when an Anabaena culture has time for the induction of rhodanese and other enzymatic pathways, the rate constants for cyanide biodegradation increase. Because the Anabaena in an operating cyanide-treatment process would be acclimated to a steady-state cyanide concentration, the rate constants for cyanide biodegradation would likely be faster than those listed on Table 3.

## METHANE PRODUCTION RATES

### Rate Constants for Methane Production

For the methane production data shown on Figure 6, an unacclimated Anabaena culture ($X_o$=1146 ug chl/L) produced 14 ug $CH_4$/L (gas phase) in 1.75 hours when the initial total cyanide concentration in the vial was 31.2 ug CN/L (water phase). This amount of methane production corresponded to the reduction of 11.0 ug CN/L (water phase) by nitrogenase in 1.75 hours. The ability of nitrogen-fixing cyanobacteria to reduce cyanide concentrations

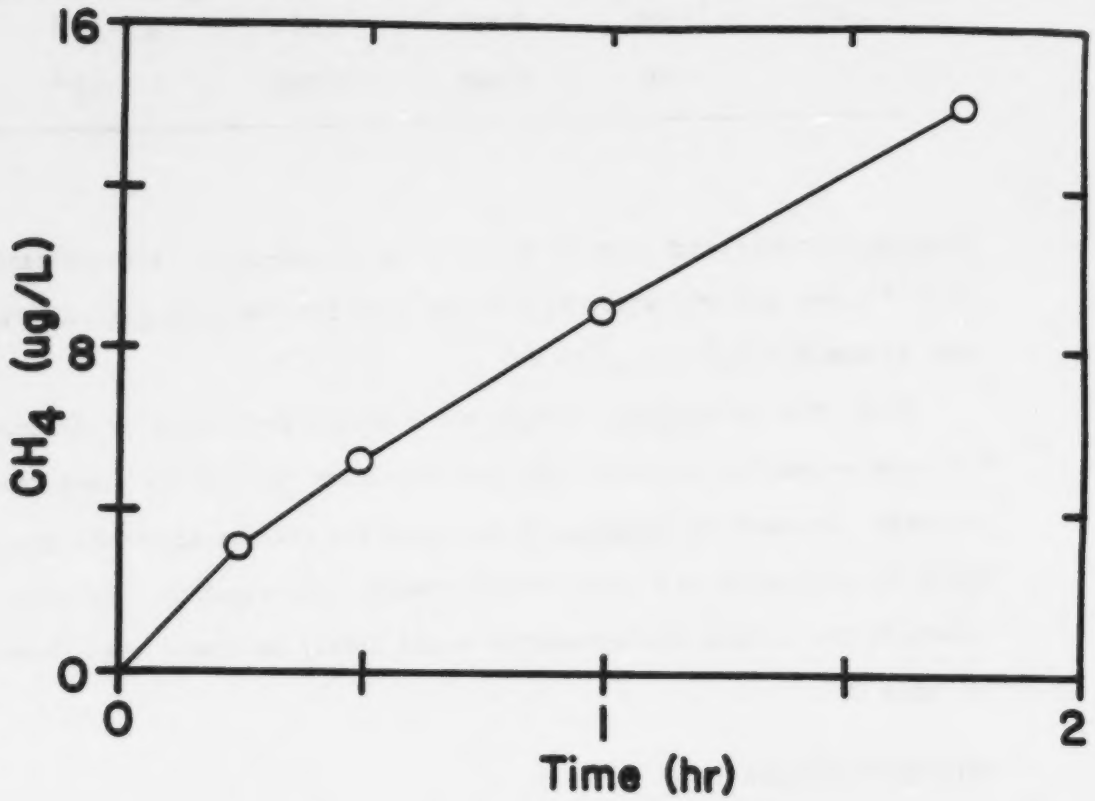


Figure 6. Increase in headspace methane concentration with time. For the illustrated batch test, the initial cyanide concentration was 31.2 ug CN/L.

below 20 ug/L may be significant, because no cyanide destruction process has demonstrated the ability to reduce total cyanide concentrations to levels less than 25 ug/L (Brunker, 1980). Thus, nitrogen-fixing cyanobacteria may be well suited for use in the secondary or tertiary treatment of cyanide wastes.

The suitability of utilizing the nitrogen-fixing capacity of cyanobacteria to treat small concentrations of cyanide can be related to the mechanism by which nitrogenase synthesis is regulated. Nitrogenase synthesis is induced by the lack of ammonia (Stewart, 1977; 1980). Thus, the intracellular concentration of nitrogenase is not a function of substrate concentration ($N_2$ or HCN), but is a function of product concentration (ammonia). By promoting the active growth of cyanobacteria, ammonia is kept in short supply, which results in the maintenance of maximal levels of nitrogenase (Stewart, 1977), regardless of cyanide concentration. In constrast to nitrogenase, the synthesis and maintenane of high intracellular concentrations of rhodanese is a function of cyanide concentration (Atkinson, 1975; Atkinson, et al., 1975; Brunker, 1980). The ability to maintain maximal activity levels of nitrogenase at low cyanide concentrations implies that nitrogenase may be more effective in the removal of low levels of cyanide than cyanide-induced enzymatic pathways.

The determination of the rate constant for the reduction of cyanide by nitrogenase required the calculation of the mixed second-order rate constant for the overall removal of cyanide from the vial. As the pH in the vial was 10, the value of mixed second-order rate constant for the total cyanide removal rate ($K_b$) calculated from equation (17) was $4.33 \cdot 10^{-4}$ L/(ug chl hr). This $K_b$ value was used in equations (13) and (14) to calculate the mixed

second-order rate constant ($K_n$) for the reduction of cyanide by nitrogenase. For the methane production shown in Figure 6, the calculated $K_n$ value was $2.58 \cdot 10^{-4}$ L/(ug chl hr).

A quick comparison of the calculated $K_n$ and $K_b$ values would suggest that for an initial cyanide concentration ($S_o$) of 31.2 ug CN/L, the rate of HCN reduction by nitrogenase accounted for 60% of the total cyanide removal rate. One problem with this comparison is that the calculation of $K_b$ from equation (17) assumes that $S_o$ is approximately 3000 ug CN/L. Because the value of $K_b$ was found to increase with decreasing HCN concentration, the use of equation (17) in the analysis of above methane production experiment may greatly underestimate $K_b$. Fortunately, the $K_n$ value obtained from equations (13) and (14) was relatively insensitive to changes in $K_b$. For inputted total cyanide biodegradation rate constants ($K_b$) ranging from $2.58 \cdot 10^{-4}$ (all cyanide removal due to nitrogenase) to $1.0 \cdot 10^{-3}$ L/(ug chl hr) (only 5% of original cyanide remained in reactor after 1.75 hr), the $K_n$ values obtained from equations (13) and (14) only varied by a factor of 2 (Figure 7). For the inputted range of $K_b$ values, the ratio of nitrogenase activity to total cyanide biodegradation rates ($K_n/K_b$) ranged from 0.39 to 1.00 (Figure 8). These calculations suggest that the reduction of HCN to methane by nitrogenase is a significant mechanism of cyanide biodegradation for unacclimated Anabaena cultures exposed to low cyanide concentrations ($S_o$=31.2 ug CN/L).

As the initial concentration of cyanide increased, the mixed second-order rate constant for nitrogenase activity ($K_n$) appeared to decrease. For a methane production experiment with an initial cyanide concentration ($S_o$) of 400 ug CN/L and a pH of 9.9, the calculated $K_n$ value was $2.62 \cdot 10^{-6}$ L/(ug chl hr). This $K_n$ value was 2 orders of magnitude slower than the $K_n$

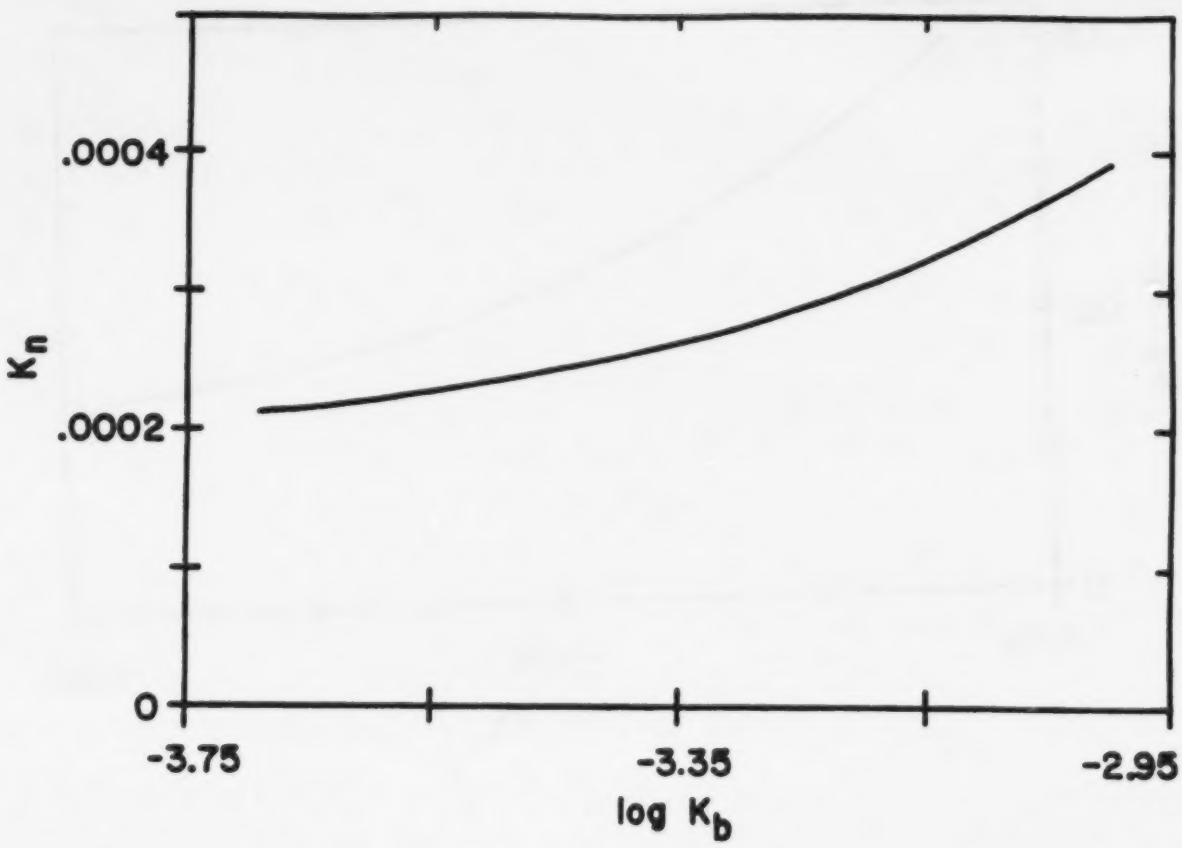


Figure 7. Sensitivity of $K_n$ obtained from equations (13) and (14) to inputted $K_b$ values. Both parameters have units of L/(ug chl hr).

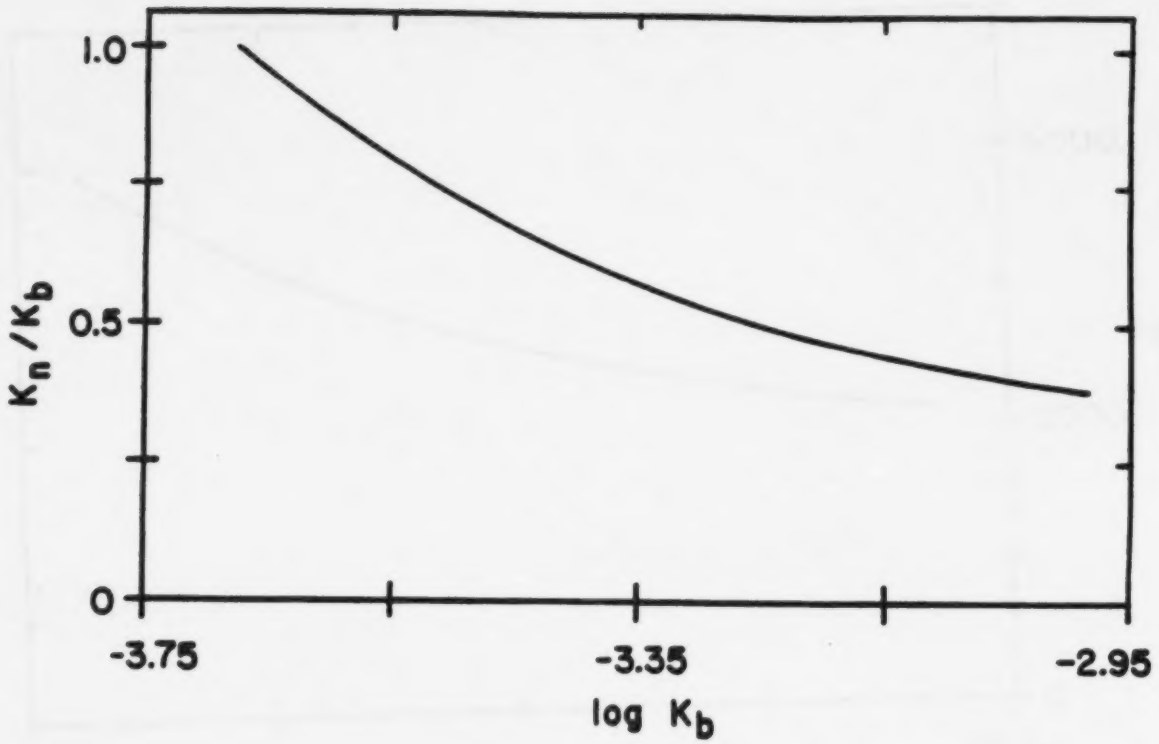


Figure 8. Sensitivity of the ratio between $K_n$ and $K_b$ to inputted $K_b$ values. Both parameters have units of L/(ug chl hr).

value calculated when $S_o$ was 31.2 ug CN/L. The decrease in $K_n$ with increasing $S_o$ was probably due to HCN inhibition of the ATP generating pathways in the heterocysts, because cyanide at the experimental concentrations has no effect on the rate of electron flow through nitrogenase (Li, et al., 1982). Thus, the effect of cyanide concentrations on $K_n$ may diminish with acclimation.

Comparison of Methane Production with Nitrogen Fixation

The observed rate of HCN reduction (methane production) by nitrogenase was compared to literature values of dinitrogen ($N_2$) reduction rates. The dinitrogen reduction rates were converted to HCN reduction rates based on three assumptions. First, the rate of electron flow through nitrogenase is independent of HCN concentration, so that the rate of electron flow for nitrogen fixation is assumed equal to electron flow for HCN reduction (Li, et al., 1982). This is a reasonable assumption for the experimental conditions described above. Second, all of the cyanide inside the heterocyst is in the form of HCN. This is a reasonable assumption, because the pH inside of a heterocyst is 7.3 (Stewart, 1977), two pH units below the $pK_a$ for HCN. Third, for each mole dinitrogen reduced there is the simultaneous reduction of 2 protons to make 1 mole of hydrogen gas, i.e., 8 electrons must flow through nitrogenase for each mole of $N_2$ fixed. This is a conservative estimate of the moles of $H_2$ produced per mole of $N_2$ reduced by nitrogenase (Burris and Peterson, 1978; Lean, et al., 1978; Postgate, 1982; Fay, 1983; Bothe, et al., 1984).

Ramos, et al., (1987) reported that an Anabaena culture had a maximum specific dinitrogen reduction rate of 30 umole $N_2$/(mg chl hr). Based on the above assumptions, this corresponded to a calculated maximum specific HCN reduction rate (k) of 1.08 ug HCN/(ug chl hr). This maximum rate was adjusted

to account for the effect of ambient HCN concentrations by the following modified Monod equation:

$$r_{HCN} = \frac{k\ S}{K_m + S} \tag{18}$$

in which $r_{HCN}$ is the specific HCN reduction rate [ug HCN/(ug chl hr)], k is the maximum specific HCN reduction rate [1.08 ug HCN/(ug chl hr)], S is the ambient HCN concentration (ug HCN/L), and $K_m$ is the Michaelis-Menten constant for the production of methane from HCN by nitrogenase (121,500 ug HCN/L) as reported by Li, et al., (1982). This $K_m$ value included both the effect of enzyme affinity for HCN and of $H_2$ production on $r_{HCN}$. Because $K_m$ is much larger than the ambient cyanide concentrations used in the methane production experiments, equation (18) can be reduced to

$$r_{HCN} = \frac{k}{K_m} S \tag{19}$$

in which $k/K_m$ has the value $8.9 \cdot 10^{-6}$ L/(ug chl hr). Equation (19) is equivalent to the following rearrangement of equation (8):

$$\frac{1}{X_o}\frac{dP}{dt} = K_n\ S \tag{20}$$

in which $K_n$ is the experimentally-derived mixed second-order rate constant for the production of methane by nitrogenase [L/(ug chl hr)]. The calculated $k/K_m$ value was compared to the experimentally-derived $K_n$ values.

The observed values of $K_n$ ranged from $2.62 \cdot 10^{-6}$ L/(ug chl hr) when $S_o$ was 400 ug CN/L to $2.58 \cdot 10^{-4}$ L/(ug chl hr) when $S_o$ was 31.2 ug CN/L. The calculated $k/K_m$ value of $8.9 \cdot 10^{-6}$ L/(ug chl hr) lies between the two observed $K_n$ values. Thus, the observed rates of methane production were reasonable compared to known <u>in vivo</u> nitrogen-fixation rates and <u>in vitro</u> HCN reduction

rates.

The observed $K_n$ value for $S_o$=31.2 ug CN/L was almost 30 times larger than the calculated $k/K_m$ value. This suggests that Anabaena may reduce low concentrations of HCN at a more rapid rate than dinitrogen. Such a conclusion agrees with the observation by Li, et al., (1982) that the activation requirements for nitrogenase to reduce HCN are lower than the activation requirements to reduce dinitrogen. Because lower activation energies usually translate into faster kinetics, nitrogenase will preferentially reduce HCN. Thus, if nitrogenase's requirements for ATP and electrons can be continuously satisfied, then the enzymatic apparatus in Anabaena normally responsible for nitrogen-fixation may play an important role in determining the rate at which low concentrations of cyanide are biodegraded in a treatment process. In the methane production experiments with $S_o$=31.2 ug CN/L, the rate of nitrogenase activity was responsible for at least 39% of the total cyanide biodegradation rate (Figure 8).

## APPLICATION OF KINETIC DATA TO CYANIDE TREATMENT

The purpose of this section is to predict the strength of a cyanide waste that can treated by a process utilizing suspended cultures of nitrogen-fixing Anabaena. This prediction will assume steady-state conditions in a once-through competely-mixed reactor. Because the kinetic data collected in this study were not obtained under steady-state conditions, application of the following discussion to the design of an operating cyanide treatment process should be done with great caution.

Assuming mixed second-order biodegradation kinetics and ignoring volatilization, the mass balance equation for cyanide in a completely-mixed once-through reactor is as follows:

$$V \frac{dS}{dt} = Q S_i - Q S - K_b X S \qquad (21)$$

in which V is the volume of the reactor (L), S is the concentration of cyanide in the effluent and in the reactor (ug CN/L), $S_i$ is the cyanide concentration in the influent (ug CN/L), t is time (hr), Q is the volumetric flow rate through the reactor (L/hr), X is the concentration of Anabaena in the reactor (ug chl/L), and $K_b$ is the mixed second-order rate constant for the biodegradation of cyanide by Anabaena [L/(ug chl hr)]. Under steady-state conditions (i.e., dS/dt=0 and dX/dt=0), the influent concentration can be described by

$$S_i = S [1 + K_b X \Theta] \qquad (22)$$

in which $\Theta$ is the hydraulic retention time (hr) and is equal to V/Q. Thus, by assigning values of S, $K_b$, X and $\Theta$, the strength of the cyanide waste can be estimated from equation (22).

In predicting the influent cyanide concentrations that can be treated by an Anabaena process, the following parameters were assumed:

| | |
|---|---|
| pH = 9.5 | temperature = 25°C |
| X = 500 ug chl/L | S = 300 ug CN/L |
| $\Theta$ = 120 hr | $K_b$ = 0.00022 L/(ug chl hr) |

The values of the above parameters are considered to be conservative. The value of S is an order of magnitude smaller than the cyanide concentration at which $K_b$ was determined. The above $K_b$ value assumes an unacclimated Anabaena culture, under steady-state conditions $K_b$ should be larger. The biomass concentation (X) is an order of magnitude smaller than the highest concentration observed in the experimental reactor. A $\Theta$ of 120 hr (5 days) provides a safety factor of 10 above the average net specific growth rate for

Anabaena. Substitution of the above parameters into equation (22) yields a $S_i$ of 4260 ug CN/L (4.26 mg CN/L). Thus, if a steady-state Anabaena concentration of 500 ug chl/L can be maintained in a chemostat with a hydraulic retention time of 5 days, then the reactor should be able to reduce an influent cyanide concentration of 4.26 mg/L to an effluent concentration of 0.3 mg CN/L, a 93 percent reduction. As the rate of cyanide biodegradation is first-order with respect to cyanide concentration (equation 22), similar removal efficiencies should be observed for lower influent cyanide concentrations.

The above calculations predict that a chemostat with a hydraulic retention time ($\theta$) of 5 days is required to achieve a 93% reduction in cyanide. For some cyanide-waste generators, the costs associated with the construction of a reactor large enough to hold the volume of wastes generated in 5 days may be prohibitive. However, the volumetric size of a cyanobacteria reactor could be reduced by using methods to make the mean cell residence time ($\theta_c$) larger than the hydraulic retention time ($\theta$).

One possible method for increasing $\theta_c$ involves the recycling of cyanobacteria biomass in the effluent line to the reactor. The activated-algae reactor was originally proposed by McGriff and McKinney (1971). The success of the activated-algae reactor is dependent on the ability to separate algal cells from the reactor effluent by settling. Despite the presence of gas vesicles, cyanobacteria will sink when their growth is limited by fixed nitrogen availability (Klemer, et al., 1982). Because nitrogen-fixing cyanobacteria only produce one mole of ammonia per cyanide reduced instead of the two obtained from dinitrogen, the availability of nitrogen may limit cyanobacteria growth rates in reactors used to treat cyanide wastes. While

cyanobacteria may sink, the potentially-slow settling velocities of the filamentous nitrogen-fixers might require clarifiers with large surface areas. Thus, the reduction in construction costs for the activated-algae reactor compared to a chemostat could be partially negated by the construction costs for a clarifier.

Another method of increasing $\Theta_c$ would be the use of attached-growth reactors instead of suspended-growth chemostats. By growing cyanobacteria on attached media steady-state concentration of biomass becomes less dependent on hydraulic flow rates. One problem with a cyanobacteria biofilm reactor is assuring that sufficient light intensity reaches all of the cyanobacteria biofilms. If the light availability problem can be solved, then the combination of the large $\Theta_c$ associated with biofilm reactors and the ability of nitrogenase to detoxify trace-concentrations of cyanide suggests that a nitrogen-fixing cyanobacteria biofilm reactor would be well suited for the secondary or tertiary treatment of cyanide wastes.

In addition to providing adequate mean cell retention times, another concern is protecting the cyanobacteria from fluctuations in cyanide concentration. Despite the acclimation of a microbial process to a given cyanide concentration, small short-term increases in cyanide concentrations can be inhibitory to the microorganisms and, thus, disrupt the microbial process (Gaudy, et al., 1982). One means of protection is to dampen the magnitude of the influent cyanide concentration fluctuations by primary treatment of the cyanide wastes before biological treatment. Primary treatment of the cyanide wastes would also reduce the cyanide load to the cyanobacteria process. Thus, the steady and small concentrations of cyanide in the effluent of an alkaline-chlorination or other primary treatment process

would be conducive to the maintenance of cyanobacteria in a secondary treatment process. Utilization of nitrogen-fixing cyanobacteria to detoxify the last fraction of cyanide, instead of attempting to treat all of the cyanide by the primary process, should result in lower operating costs for larger cyanide treatment facilities.

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